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A Remote Controlled Valve in Liposomes for Triggered Liposomal Release

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In order to reduce the toxicity and increase the efficacy of drugs, there is a need for smart drug delivery systems. Liposomes are one of the promising tools for this purpose. An ideal liposomal delivery system should be stable, long-circulating, accumulate at the target site and release its drug in a controlled manner. Even though there have been many developments to this end, the dilemma of having a stable liposome during circulation but converting it into a leaky structure at the target site is still a major challenge. So far, most attempts have focused on destabilizing the liposome in response to a specific stimulus at a target site, but with limited success. Our approach is to keep the stable liposome but build in a remote-controlled valve as a new release mechanism, instead. The valve is a pore-forming bacterial membrane protein. It has been engineered such that, after being reconstituted into the liposomes, its opening and closing can be controlled on command by the ambient pH, light or a combination of both. In addition, a much higher degree of flexibility for fine-tuning of the liposome's response to its environment is achieved.

Keywords triggered liposomal release, mechanosensitive channel of large conductance (MscL), liposomes, drug delivery, pH induced release, light induced release, channel protein modifications

Introduction

After their discovery in 1965, liposomes became a promising tool for drug delivery (Bangham et al., 1965). Since then, there have been many developments in liposomal composition, efficient drug encapsulation and retention, stability and targeting (Woodle and Lasic, 1992). However, in most cases the amount of drug passively released from such ideal long-circulating, stearically stable liposomes was not enough to show a therapeutic effect. To this end, more research efforts were directed towards triggered release in response to a specific stimulus at a target site. It has been hypothesised that enhanced release at the target site will significantly improve the specificity and

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efficacy of a liposomal drug (Bally et al., 1998; Drummond et al., 1999; Fenske et al., 2001).

There have been different strategies used for triggering liposomal release based on destabilization of the liposome bilayer. Formation of channels and defects in the bilayer, lamellar-micellar or lamellar-hexagonal phase transition, lipid phase separation and liposome fusion are some examples (Thomas et al., 1992; Chu et al., 1994; Drummond et al., 2000; Meers, 2001; Asikan et al., 2002). All these efforts have been focused on the lipid components of the liposome, and, for the most part, met with limited success (Guo and Szoka, 2003).

As an alternative, in this article we present an engineered bacterial channel protein as a remote-controlled nano-valve in stearically stable liposomes for triggered release of the liposomal content on command (Kocer et al., 2005, 2006).

Channel proteins are excitable pores embedded in cell membranes that, by opening and closing, allow the flow of ions across the membrane. One of these proteins, the Mechanosensitive channel of large conductance (MscL) from *Escherichia coli*, is very attractive as a release valve in liposomal delivery systems. First of all, it keeps its functionality when it is reconstituted into artificial lipid bilayers without requiring any other cellular components. Second, it is unique for its large and non-selective pore that allows the passage not only of ions but also small molecules up to 6.5 kDa (Van den Bogaart et al., 2007). Last but not least, it is one of the best studied channel proteins.

In nature, this homopentameric protein is embedded in the cytoplasmic membrane (Berrier et al., 1989; Blount et al., 1996) and protects the bacterial cell against severe osmotic shocks (Levina et al., 1999). Under hypo-osmotic conditions, cell turgor leads to tension in the membrane and an altered lateral pressure profile, which, in turn, triggers large conformational changes on the channel (Perozo et al., 2002). The result is a 3 nm non-selective pore through which molecules can flow in order to balance the osmotic difference between the interior and exterior of the cell (Cruickshank et al., 1997, Ajouz et al., 1998). However, in order to operate it more easily in liposomal delivery systems, we reengineered its opening mechanism. Instead of tension, we made it responsive to triggers that can be found or generated at the drug delivery sites such as ambient pH and light.

In order to do so, a critical intrinsic property of the channel was altered. Although normally the channel opens in response to tension, its opening is influenced also by the polarity of its hydrophobic constriction zone (Anishkin et al., 2005). An increase in the polarity or hydrophilicity of the 22nd amino acid, located in this part of the protein, shifts the tension threshold for channel opening to lower values (Yoshimura et al., 1999). In extreme cases, the channel opens even in the absence of tension (Yoshimura et al., 2001). Here, on the basis of this charge-induced opening principle, we rationally designed sulfhydryl-reactive chemical modulators and covalently and specifically attached them to engineered cysteines in the pore. When placed in this hydrophobic region of the channel, these modulators would alter the hydration and open the channel, by creating charge but only in response to an external stimulus (Fig. 1).

Materials and Methods

Detailed procedures for the chemical synthesis, protein isolation, chemical modification of the protein and efflux experiments can be found in Kocer et al. (2006, 2007).

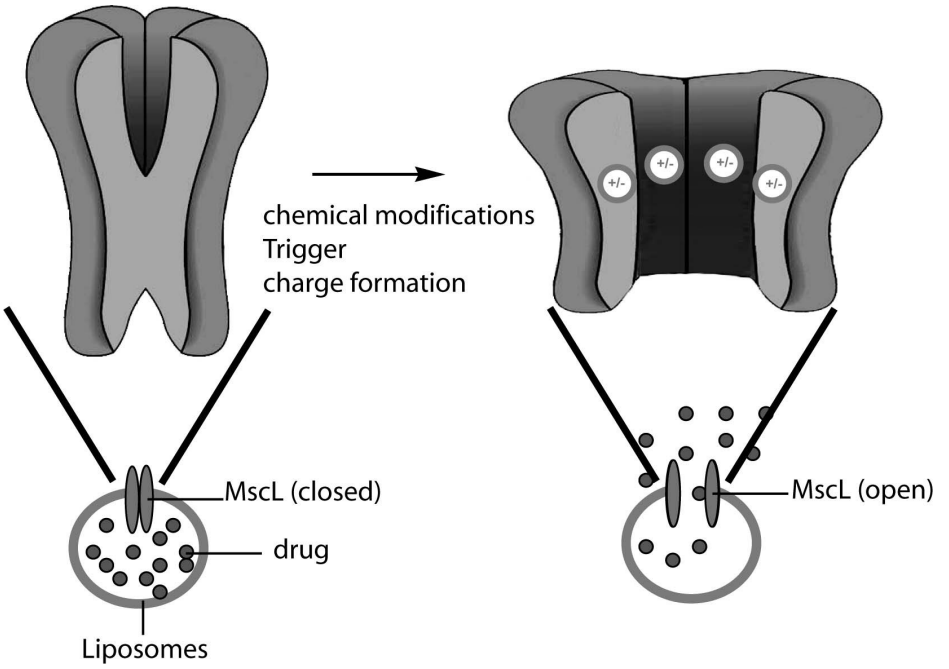


Figure 1. Principle of triggered liposomal release through an engineered channel protein. The engineered channel opens when its hydrophobic pore region is charged in response to a desired stimulus. (Only 4 of the 5 subunits are shown in the conceptual drawing).

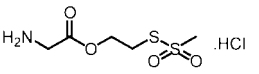
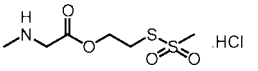
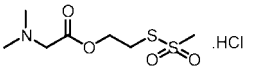
Results and Discussion

pH-Induced Liposomal Release

In order to release liposomal content in response to ambient pH, we modified MscL by coupling charge-induced channel opening of MscL to ambient pH. *In vivo* this parameter varies depending on the health status of the surrounding tissue and/or cellular compartment. Around solid tumors, sites of inflammation, endosomes and lysosomes the pH is lower (pH 6.8 to 5.0) compared to normal physiological conditions (pH 7.4) (Ulbrich and Šubr, 2004).

In order to achieve pH-induced liposomal release, the channel was modified with specifically designed pH-responsive chemical modulators (Table 1). Modified channels were, then, incorporated in liposomes consisting of DOPC: Cholesterol: DSPE-PEG2000 (70: 20: 10 *M* ratio) using a detergent-mediated reconstitution method in the presence of calcein, a self-quenching fluorescent dye (Rigaud et al., 1995). The pH-induced, channel-mediated release has been followed under iso-osmotic conditions in a straightforward liposomal efflux assay, using fluorescence changes upon the release of calcein. Calcien fluorescence is quenched in the liposome interior due to the high concentration of the dye but it is highly fluorescent at the lower concentrations occurring upon release from the liposome. Briefly, after chromatographic removal of external dye, the resulting proteoliposomes (average size 200 nm, as determined by dynamic light scattering) were analyzed for channel activity at different pH's. Channel openings led to an increase in fluorescence

Table 1
pH responsive chemical modulators

Compound number	Structure
1	
2	
3	

(Fig. 2a). Proteoliposomes containing the channel responded to pH, releasing more calcein at pH's below the pK_a of the modulator used. Controls with liposomes lacking MscL-G22C or containing unmodified MscLG22C or wild-type MscL did not show any release activity.

After proving the pH-induced liposomal release via the engineered MscL channel, the pH-sensitivity interval of the channel was tuned by varying the pKa and hydrophobicity of the modulators. For example, liposomes with MscL, which is modified with a compound with a calculated pKa of 7.35, retain their content at pH 7.4, the physiological pH for human, but start to open and release content at lower pH's (Fig. 2b).

Light-induced Liposomal Release

In order to test the activation of the MscL-containing liposomes with external stimuli, we used light as a trigger. To this end, light-responsive chemical modulators were designed, synthesized and, as before, covalently attached to MscL. The principle is that, illumination leads to a localized buildup of charge and consequent opening of the channel, hence release of the liposomal content. The modulator shown in Fig. 3a is sensitive to long-wavelength ultraviolet (UV), a wavelength range that is compatible with most biomaterials

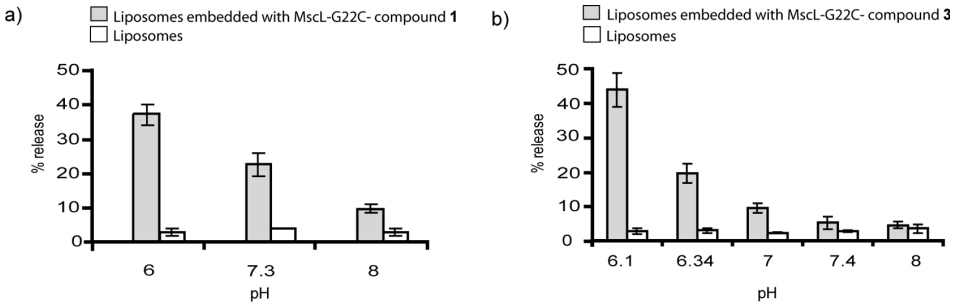


Figure 2. pH-dependent liposomal release in calcein efflux assays. Maximum release was reached within 30 min and reported here relative to the release obtained after bursting the liposomes with excess Triton X100. Error bars indicate the standard deviation from four independent experiments. a) pH- dependency of liposomes embedded with MscL-G22C-compound **1**. b) pH- dependency of liposomes embedded with MscL-G22C-compound **3**.

(Bochet, 2002). Illumination at $\lambda > 300$ nm results in photolysis of the protective group, and leaves channel-bound acetates, which are negatively charged above pH 4.0. This results in channel opening and release of the liposomal content (Fig. 3b).

A further control over liposomal release was obtained by operating the channel activity in a reversible way. To this end, a photo-induced switch, based on a spiropyran core attached to a cysteine-selective iodoacetate moiety was synthesized and coupled to MscL. Upon irradiation at 366 nm, photochemical ring opening takes place, resulting in a charged zwitterionic merocyanine structure (MC), which opens the channel. Exposure to visible light ($\lambda > 460$ nm), on the other hand, results in the reverse, ring- closing reaction, restoring the original uncharged spiropyran state (SP), which closes the channel (Fig. 3 of Kocer et al., 2005).

Light-triggered pH-induced Liposomal Release

Further control over the timing, location and amplitude of the liposomal release was obtained through the modification of MscL with caged modulators, in which a photoremovable group protects the pH-modulator. Liposomes with such channels thus are inert to the environmental pH until exposure to long wavelength UV, at which point pH-responsiveness is immediately realized. This additional tool results in more flexibility and precision with respect to external control over the channel opening and the associated release of molecules from liposomes (Fig. 4 of Kocer et al., 2006).

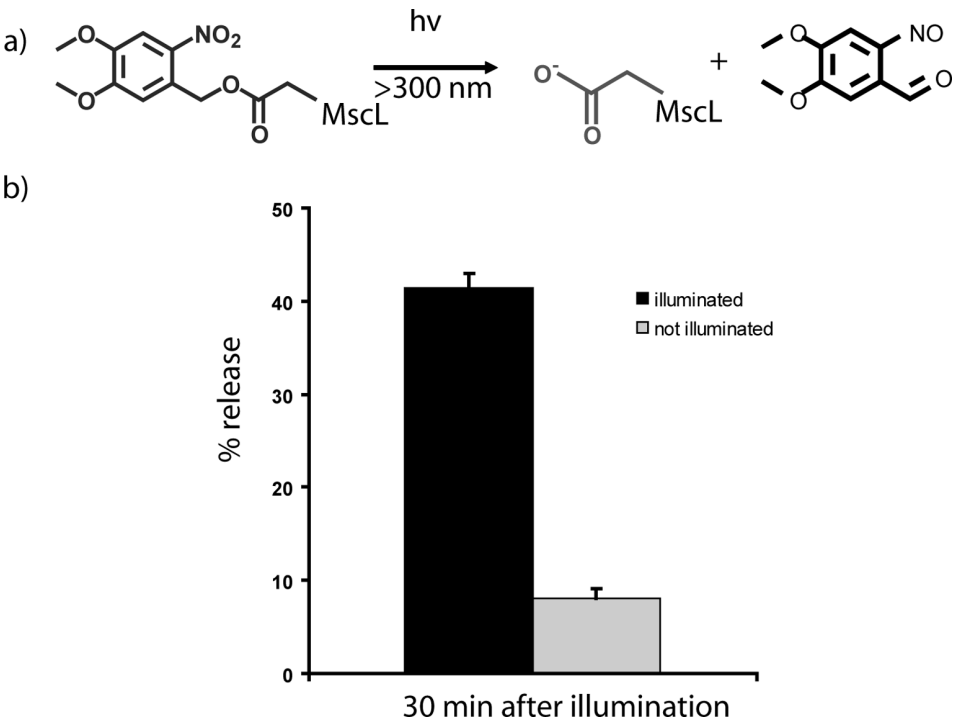


Figure 3. Light-dependent liposomal release. a) Mechanism of charge formation in MscL in response to light. b) Light-dependent liposomal release in calcein efflux assays.

Conclusions

Shortly after their discovery, Gregoriadis et al. (1974) suggested the use liposomes as drug delivery vesicles. Since then a significant amount of research has been performed to obtain an ideal drug delivery system. Such a system should have a long circulation time in the blood, accumulation at the target site and controlled drug release that matches the efficacy profile of the drug.

The long circulating and stable liposomes were obtained with the cost of a very low release profile. This dilemma of having a stable liposome structure during circulation but having a leaky structure at the target site is only now beginning to be solved by the use of specific liposome compositions in conjunction with externally applied triggers such as focused ultrasound, oscillating magnetic fields and light (Andresen, et al., 2005; Chandra, et al., 2006; Dromi et al., 2007; Huang et al., 2002; Zhang et al., 2005). So far, most attempts have been based on destabilization of the liposome structure. Our approach is to keep the stable liposome structure as it is and to reconstitute a pore-forming bacterial membrane protein into the liposome and use it as the release mechanism. The channel protein is engineered such that its opening and closing can be controlled on command. Synthetic chemistry in combination with channel biology provides a much higher degree of flexibility for fine-tuning the liposome's response to its environment. For example, the pH in the environment of a solid tumor is about 6.5. Technically, it is difficult to design liposomes that can be stable at physiological pH 7.4, but leaky at pH 6.5 (Andresen, 2005). However, we have shown that with our system it is possible to fine-tune the pH release profile of liposomes by fine-tuning of the pH response of the reconstituted channel protein.

A major challenge of the proposed method is a possible immune response to a channel protein of bacterial origin. Presently immunogenicity studies are in progress.

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